Rapamycin-Sensitive Pathway Regulates Mitochondrial Membrane Potential, Autophagy, and Survival in Irradiated MCF-7 Cells

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Abstract

Radiation-induced inhibition of rapamycin-sensitive pathway and its effect on the cellular response to radiation were studied in the human breast cancer cell line MCF-7. Both radiation and rapamycin shared molecular targets and induced similar physiologic responses. Each of these treatments increased immunostaining of mammalian target of rapamycin (mTOR) in the nucleus, and radiation led to decreased phosphorylation of its autophosphorylation site Ser2481. In addition to dephosphorylation of established mTOR downstream effectors 4E-binding protein 1 and p70 ribosomal S6 kinase, both treatments decreased the level of eukaryotic initiation factor 4G. Experiments with the potentiometric dye, JC-1, revealed an oligomycin-dependent increase in mitochondrial membrane potential following radiation or rapamycin treatment, suggesting that both lead to reversal of F0F1 ATPase activity. Both radiation and rapamycin induced sequestration of cytoplasmic material in autophagic vacuoles. In both cases, appearance of autophagic vacuoles involved the participation of microtubule-associated protein 1 light chain 3 (LC3). Transient cotransfection of green fluorescent protein-LC3 with either wild-type or dominant-negative mTOR further showed that inactivation of mTOR pathway is sufficient to induce autophagy in these cells. Finally, administration of rapamycin in combination with radiation led to enhanced mitochondria hyperpolarization, p53 phosphorylation, and increased cell death. Taken together, these experiments show that radiation-induced inhibition of rapamycin-sensitive pathway in MCF-7 cells causes changes in mitochondria metabolism, development of autophagy, and an overall decrease in cell survival.

Introduction

The serine/threonine phosphatidylinositol 3-kinase (PI3K)-related kinase, target of rapamycin (TOR), regulates protein translation, progression of cells through the cell cycle, biogenesis of ribosomes and tRNAs, and autophagy (1). In yeast and mammalian cells, TOR molecules reside in multimolecular complexes (1, 2). The first complex to be isolated from mammalian cells contains two newly discovered proteins: raptor and GβL (3–5). Raptor is thought to mediate the interaction between mammalian TOR (mTOR) and its substrates. Its interaction with the TOR signaling domain of both 4E-binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase (p70S6K) was found to play an important role in mTOR-dependent phosphorylation of these proteins (6). Interestingly, the dependence of 4E-BP1 phosphorylation on the interaction with raptor is greater than that of p70S6K (6), suggesting that cells may be able to differentially regulate various mTOR functions. The activity of the mTOR-raptor complex is sensitive to rapamycin, an immunosuppressant macrolide (3, 5). Rapamycin, in complex with FK506-binding protein 12, binds to the FK506 rapamycin-binding domain in mTOR and inhibits its functions (1). Rapamycin is thought to exert its effect on mTOR activity by inhibiting the interaction of the enzyme with its substrates and modulators rather than by inhibiting its catalytic activity (7, 8). The second multimolecular complex of mTOR contains, aside from GβL, another newly discovered protein named rictor. This complex is insensitive to rapamycin and mediates the organization of actin filaments in the cells via its interaction with protein kinase Cα (2).

As a major player in cellular homeostasis, mTOR receives signals from various pathways and molecules (1). Most importantly, components of PI3K/Akt/mTOR pathway have oncogenic versions, are capable of oncogenic transformation, or are overexpressed in many types of cancers leading to deregulation of this pathway, thereby implicating it in tumor development (9, 10). In accordance with these observations, rapamycin was found to inhibit the oncogenic transformation induced by overexpression of PI3K and Akt but not by the activity of 11 other oncoproteins (11). Rapamycin was also found to inhibit tumor growth in animal models (12). However, recent results indicate that both the role of mTOR pathway in tumorigenesis and the inhibitory effect of rapamycin on tumor growth may vary among different cells (13, 14).

The activity of mTOR pathway is affected by stress signals, such as DNA damage and decreased level of amino acids, energy-rich compounds, and oxygen (15). Here too, as discussed by Castedo et al. (16), the response of mTOR pathway to stress and its role in modulating the sensitivity of the cells to those signals is cell context dependent. Thus, treatment of 3T3 cells with DNA-damaging agents, such as etoposide, cisplatin, or mitomycin A, led to inactivation of mTOR pathway as evidenced by dephosphorylation of p70S6K and 4E-BP1 (17). On the other hand, ionizing irradiation led to activation of mTOR pathway in rat mammary-derived fibroblasts (MM3MG) and mouse embryonic fibroblasts (MEF) cells (18, 19). However, whereas the activity of mTOR pathway in irradiated MM3MG cells was associated with activation of a defense program, and exposure to rapamycin led to increased sensitivity to radiation, in irradiated MEF cells the activity of mTOR pathway was associated with increased immunolocalization of...
mTOR in the nucleus, phosphorylation of p53, activation of caspase-3, and cell death. Exposure to rapamycin protected the cells against radiation damage (19). Similarly, in HeLa cells, fusion between HIV-1 Env-expressing cells and those expressing CD4/CXCR4 led to cell death that was preceded by increased immunolocalization of mTOR in the nucleus, phosphorylation of p53, collapse of mitochondrial membrane potential (ΔΨm), and apoptosis. Here too, p53 phosphorylation, collapse of ΔΨm, and cell death were diminished by treatment with rapamycin (20).

It is therefore clear that rapamycin-sensitive pathway affects growth of cancer cells and their response to anticancer treatments; these effects, however, differ among cells. Therefore, understanding the mechanisms that govern this pathway in tumorigenesis and in response to stress in various cancer cells is important for its successful engagement in cancer therapy.

The goals of this work were to assess radiation-induced changes in rapamycin-sensitive pathway in MCF-7 cells and to determine their effect on the cellular response to radiation damage. Our experiments indicated that radiation-induced inhibition of rapamycin-sensitive pathway regulates mitochondria hyperpolarization, autophagy, and p53 phosphorylation; most importantly, inhibition of this pathway is detrimental to cell survival.

Materials and Methods

Reagents. Rapamycin, Complete Mini EDTA-free protease inhibitor cocktail tablets, and transfection reagent FuGENE 6 were from Roche (Indianapolis, IN); calyucin was from Alexis Biochemicals (San Diego, CA); 7-methyl-GTP-Sepharose beads were from Pharmacia Biotech (Piscataway, NJ); VectaShield-mounting medium was from Vector Laboratories (Burlingame, CA); nonimmune goat IgG was from Jackson Immunoresearch (West Grove, PA); anti–IE-BP1 was from StressGen (Victoria, British Columbia, Canada); anti–eukaryotic initiation factor 4G (eIF4G) and anti–FRAP (N-19) raised against a peptide mapping at the NH2 terminus of mTOR were from Santa Cruz Biotechnology (Santa Cruz, CA); and anti–eukaryotic initiation factor 4E (eIF4E) was from BD Transduction Laboratories (San Diego, CA). Antibodies against p70S6K, p-53, and their phosphorylated forms were from Cell Signaling Technology (Beverly, MA). Secondary antibodies conjugated to Alexa Fluor 488 or 546 and the ΔΨm probe JC-1 were from Molecular Probes (Eugene, OR). Oligomycin, valinomycin, carbonyl cyanide p-chlorophenylhydrazone (CCCP), and anti–hemagglutinin (HA) antibodies were from Sigma-Aldrich (St. Louis, MO). Rabbit anti–light chain 3 (LC3) and LC3 construct in a green fluorescent protein (GFP) fusion-protein expression vector were a generous gift from Dr. T. Yoshimori (National Institute for Basic Biology, Okazaki, Japan; ref. 21). pcDNA1-HA-tagged mTOR wild-type (WT) and dominant-negative (N234K) constructs as well as pcDNA1 empty vector were a generous gift from Dr. K. Yonezawa (Biosignal Research Center; Kobe University, Kobe, Japan; ref. 22).

Cell culture. MCF-7 (human breast adenocarcinoma) cells from American Type Culture Collection (Manassas, VA) were plated at a density of 3 \times 10^4 per cm², maintained, and irradiated 48 hours after plating as described before (23). Rapamycin was added from 1 mg/mL stock in DMSO. Samples were heated at 95°C for 10 minutes and cleared by centrifugation at 10,000 \times g. Protein content was determined with a bicinchoninic acid reagent, and equal amounts of protein from each sample were analyzed by Western blotting. Treatment-induced changes in the level of proteins were estimated as described above, and values of integrated light intensity were used for calculation of treatment-induced changes in the ratio of eIF4E to eIF4G.

Cell lysis and Western blotting. Cells were washed with ice-cold DPBS and collected in buffer containing 150 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), 2% SDS, 1.7 mmol/L EDTA, 1.5 mmol/L sodium orthovanadate, 50 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 10 mmol/L sodium β-glycerophosphate, 1.5 μg/mL pepstatin, and Roche anti-protease cocktail. Samples were heated at 95°C for 20 minutes and cleared by centrifugation at 10,000 \times g. Protein content was determined with a bicinchoninic acid reagent, and equal amounts of protein from each sample were analyzed by Western blotting. Treatment-induced changes in the level of proteins were estimated as described above, and values of integrated light intensity were used for calculation of treatment-induced changes in the ratio of phosphorylated proteins to their nonphosphorylated forms. Equal loading was ensured by measuring the absorbance at 520 nm of Ponceau S extracted with DPBS from individual strips of a twin run. The absorbance of the extracted Ponceau S was linear between 10 and 40 μg total loaded proteins, a range that included the amount of proteins analyzed in our experiments.

Measurement of mitochondrial membrane potential. Changes in ΔΨm were detected with the potentiometric dye JC-1 (25). Using fluorescence microscopy, we verified that JC-1 fluorescence emanates from mitochondria and that their rod shape was...
maintained throughout the experiment. Oligomycin (50 μmol/L) and CCCP (5 μg/mL) were added 30 minutes before JC-1. Valinomycin (100 nmol/L) was added 1 hour before JC-1 (1.5 μmol/L) at 37°C and 5% CO2; the cells were rinsed with DPBS, removed by trypsinization, collected at 200 × g, and resuspended in growth medium containing 25 mmol/L HEPES without phenol red. Green and red fluorescence emission was detected with 530/30 and 585/42 bandpass filters, respectively, from 10^4 cells illuminated with blue (488 nm) excitation light in a FACSCalibur from Becton Dickinson (San Jose, CA) using CellQuest software. The red-to-green fluorescence ratio for individual cells was calculated using FlowJo software (Tree Star, Inc., San Carlos, CA).

**Statistical analysis.** Statistical analysis of the differences among various treatment and control groups was done using Student’s t test. P < 0.05 was considered statistically significant.

**Results**

Radiation affects the rapamycin-sensitive pathway. To evaluate the effect of radiation on rapamycin-sensitive pathway, we first determined whether the mTOR molecule itself is modulated by radiation. As depicted in Fig. 1A, radiation did not lead to a significant change in mTOR level; however, it did alter mTOR immunolocalization (Fig. 1B). In the unirradiated population, two types of cells could be discerned: one in which the intensity of nuclear immunostaining was lower than that of the cytoplasm and one in which it was comparable with that of the cytoplasm. However, within the irradiated population, very few nuclei showed fluorescence intensity that was lower than the cytoplasm, and a new population with bright nuclear fluorescence emerged. This phenomenon was reflected in the measured average nuclear fluorescence intensity in irradiated and control cells (Fig. 1C).

Intriguingly, a dose-dependent increase in nuclear immunostaining of mTOR was also induced by rapamycin (Fig. 1B and C), suggesting that the similar effect of radiation may reflect inhibition/alteration of mTOR functions. Replacing the primary antibody with nonimmune goat IgG or incubating the antibody with the immunogenic

![Figure 1.](https://www.aacrjournals.org/doi/10.1158/0008-5472.CAN-05-1798)
peptide before immunostaining abrogated the fluorescence signal (see Supplementary Data). In addition, a similar increase in mTOR nuclear immunostaining following radiation and rapamycin treatment was observed when immunostaining was done with a monoclonal antibody (26E3). This antibody was raised against a synthetic peptide consisting of amino acids 230 to 240 at the NH2 terminus of mTOR and was characterized by Zhang et al. (ref. 27; see Supplementary Data).

In addition to its effect on mTOR immunolocalization, rapamycin led to a slight but reproducible decrease in mTOR level (Fig. 1A). mTOR autophosphorylation at Ser2481 is regarded as a reporter of its catalytic activity (28), and although rapamycin did not lead to dephosphorylation of Ser2481 (28), it inhibited its rephosphorylation following addition of phenylephrine to serum-starved cells (29). Serum starvation and hypoxia, which lead to dephosphorylation of mTOR downstream effectors, also decrease autophosphorylation of Ser2481 (28, 30). Similarly, our experiments showed that radiation led to a decreased phosphorylation of mTOR at its Ser2481 (Fig. 2A) as well as to dephosphorylation of its downstream effectors p70S6K and 4E-BP1 (Fig. 2B).

Dephosphorylation of 4E-BP1 was manifested by disappearance of its slowest migrating forms and a comparable increase in the level of its fastest migrating form. Dephosphorylation of p70S6K was apparent from its increased downshift migration and from the decreased level of its phosphorylated isoforms. Protein dephosphorylation was noted by 6 hours following radiation, and by 48 hours, a measured 3-fold decrease in the ratio of p70S6K (P-Thr389) to total p70S6K was accompanied by a reduction in the total level of p70S6K. The effect of rapamycin on the dephosphorylation of p70S6K and 4E-BP1 developed faster than radiation and was clearly shown by 1 hour following treatment. Rapamycin-induced dephosphorylation was saturating at 10 nmol/L, and the slight dose-dependent rephosphorylation of Thr389 and Thr421/Ser424 seen

![Figure 2](image-url)
Inhibition of mTOR Pathway in Irradiated MCF-7 Cells

at 48 hours (Fig. 2B, long exposure) may have resulted from the reported ~ 10 hours half-life time of rapamycin in cell culture (31). As manifested by the overexposed autoradiograms in Fig. 2B, the effect of rapamycin on dephosphorylation of p70S6K was more pronounced than that of radiation. The partial inhibition of p70S6K in irradiated cells may therefore explain why radiation does not lead to dephosphorylation of S6 (Fig. 2C). Relevant to this phenomenon is the finding that phosphorylation of p70S6K by mTOR is less dependent on the interaction of p70S6K with raptor than that of 4E-BP1, and disruption of their interaction with raptor in vitro can abolish the phosphorylation of 4E-BP1 while maintaining ~20% of the phosphorylation of p70S6K (6). Therefore, unlike rapamycin, various stress signals may lead to a differential inhibition of rapamycin-sensitive mTOR functions and consequently to readouts that are not necessarily identical with that ensued by rapamycin.

As expected, when 4E-BP1 becomes dephosphorylated (9), the association between elf4G and elf4E was inhibited in both radiation- and rapamycin-treated cells (Fig. 2D).

Inhibition of the association between elf4G and elf4E following radiation was also reported for U937 and MEF cells (32). Interestingly, unlike the dose response of 4E-BP1 dephosphorylation that was saturating at 10 nmol/L, the rapamycin effect on the association between elf4G and elf4E was dose dependent between 10 and 50 nmol/L. We therefore determined the cellular level of elf4G and elf4E following both radiation and rapamycin treatment. It has been reported that rapamycin decreases the stability of elf4G and its level in yeast (33); however, a similar effect has not been documented in mammalian cells. Our experiments showed that rapamycin led to decreased level of both elf4G and elf4E (Fig. 2E). The decreased level of elf4G, however, was steeper than that of elf4E leading to a dose-dependent decrease in the cellular ratio of elf4G to elf4E, thus contributing to the observed dose response in the 7methyl-GTP pull-down experiments. Radiation, too, led to decreased level of elf4G but did not have a detectable effect on the level of elf4E (Fig. 2E).

Aside from dephosphorylation of p70S6K, 4E-BP1, and S6 that showed apparent saturation at 10 nmol/L, all rapamycin effects described above, including its effect on increased immunostaining of mTOR in the nuclei, were dose dependent between 10 and 50 nmol/L. Interestingly, in vitro experiments showed that rapamycin displaces raptor from its complex with mTOR in a dose-dependent manner that ranged from 1 to 1,000 nmol/L (8). The differences in sensitivity to rapamycin may therefore reflect a differential dependence of mTOR functions on its rapamycin-sensitive interaction with its modulators. Alternatively, it is possible that protein phosphatase activities induced by 10 nmol/L rapamycin are sufficiently high to lead to an apparent saturation of its dephosphorylation effect on p70S6K and 4E-BP1 (34).

Radiation and rapamycin lead to hyperpolarization of mitochondria. The experiments described above show that radiation and rapamycin share molecular targets. We therefore attempted to determine whether they lead to similar physiologic responses. Employing the potentiometric dye JC-1, we studied the effect of radiation and rapamycin on ΔΨm (Fig. 3). Both radiation and rapamycin led to hyperpolarization of mitochondria as reflected by increased mean red-to-green fluorescence ratio in JC-1-stained cells. As depicted in Fig. 3A, the fraction of cells with the highest red-to-green ratio ( ~>1.5) increased by ~5-fold following rapamycin or radiation treatment. The fact that oligomycin treatment decreased this fraction by 80% to 90% suggests that reversed FoF1ATPase activity plays a role in the observed increase of ΔΨm following radiation or rapamycin treatment. Indeed, under various physiologic conditions, mitochondrial FoF1ATPase can cleave ATP using the released energy to pump protons against their gradient outside the mitochondria matrix leading to mitochondria hyperpolarization (26). Addition of the protonophore CCCP or the potassium ionophore valinomycin led to complete mitochondria depolarization and yielded a similar red-to-green ratio in both control and treated cells, indicating that the observed differences in red-to-green ratio between control and treated cells reflect a change in ΔΨm rather than changes in the macromolecular composition of the cells. Treating cells with both rapamycin and radiation led to an additive effect on the size of the cell fraction with hyperpolarized mitochondria (red-to-green ratio > 1.5). The effect of rapamycin and radiation on mitochondria hyperpolarization was both dose dependent and time dependent. The rapamycin effect was saturating at 10 nmol/L (Fig. 3B) and was faster to develop than that of radiation (Fig. 3C). In addition, the enhancing outcome on ΔΨm following combined radiation and rapamycin treatment becomes statistically significant by 48 hours following initiation of treatment (Fig. 3C).

Radiation and rapamycin lead to sequestration of cytoplasmic material in autophagic vacuoles. The process of autophagy involves the sequestration of portions of the cytosol by cytoplasmic membrane and its later delivery into lysosomes for degradation (35). Inhibition of rapamycin-sensitive pathway is known to induce autophagy in both yeast and mammalian cells (36, 37); therefore, the inhibition of this pathway in irradiated cells may lead to autophagy. Indeed, Hamberg et al. (38) reported that radiation induced autophagy in glia cells, and we have reported that irradiation of MCF-7 cells leads to accumulation of organelles that bear morphologic similarities to autophagic vacuoles (23). Improving our experimental conditions by including paraformaldehyde in the fixative verified that both radiation and rapamycin led to sequestration of cytoplasmic material within membrane-bound autophagic vacuoles with diameter of ≥0.5 μm (Fig. 4A). In some cases, sequestration of cytosolic material by limiting double membrane and autophagosomes with residual double membrane could be detected (Fig. 4A, b and d). In other cases (Fig. 4A, a and c), a limiting single membrane was observed. The sequestered content in autophagic vacuoles consisted of ribosomes, small ~50-nm vesicles, and recognizable organelles, such as mitochondria. Sequestration of ~0.4-μm vesicles containing amorphous material and yet smaller ~50-nm vesicles was also noted (Fig. 4A, c). By 48 hours following irradiation with 10 Gy, autophagic vacuoles were present in 76% of the irradiated cells where their average fractional volume in the cytoplasm was 9 ± 1% (mean ± SE). However, in unirradiated cells, autophagic vacuoles appeared only in 22% of the population and their average fractional volume was only 2 ± 0.2% (mean ± SE). Similar to irradiation, at 48 hours after rapamycin (50 nmol/L) addition, autophagic vacuoles were found in 75% of the cells where their average fractional volume in the cytoplasm was 6 ± 0.7% (mean ± SE). A lower percentage of cells with autophagic vacuoles (56%) and lower fractional volume of autophagic vacuoles (mean ± SE, 3 ± 0.5%) were observed following treatment with 10 nmol/L rapamycin.

One of the hallmarks of autophagy is the conversion of LC3-I into the faster SDS-PAGE migrating form, LC3-II, which participates in the formation of autophagosome and is associated with their structure (21). Accordingly, autophagy is associated with increased level of LC3-II, increased ratio of LC3-II to LC3-I, and
a change in LC3 immunolocalization (21). Likewise, development of autophagy in irradiated or rapamycin-treated cells involved an increased LC3-II-to-LC3-I ratio and an overall increase in the level of LC3-II (Fig. 4B). In addition, immunostaining of LC3 revealed the typical transformation of LC3 localization during autophagy (21) from a diffused staining in control cells to a dot- and ring-like pattern in irradiated or rapamycin-treated cells (Fig. 4C).

The dose and time dependence effect of radiation and rapamycin on the development of autophagy was clearly shown in cells transiently expressing GFP-LC3 (see Supplementary Data). Although the kinetics of autophagy may have slightly varied among experiments, a clear time dependence accumulation of autophagic vacuoles was shown. Cotreatment of the cells with both rapamycin and radiation facilitated the development of autophagy at 8 hours following initiation of treatment but did not seem to have an enhancing effect at later time points.

The availability of GFP-LC3 and HA-mTOR constructs enabled us to further show that inhibition of mTOR pathway is sufficient to induce autophagy in MCF-7 cells. The cells were cotransfected with GFP-LC3 and either HA-tagged WT or dominant-negative mTOR and stained with anti-HA ensured that autophagy was monitored in cells expressing both constructs. As shown in Fig. 4D and Table 1, cotransfection with dominant-negative mTOR led to increased autophagy compared with cotransfection with WT mTOR or the empty vector.

Inhibition of rapamycin-sensitive pathway in irradiated cells leads to decreased cell survival. The experiments described above suggest that inhibition of rapamycin-sensitive pathway in irradiated cells leads to mitochondria hyperpolarization and autophagy. The following experiments were undertaken to determine how inhibition of this pathway affects the sensitivity of the cells to ionizing irradiation. Following DNA damage, p53 is a known regulator of cellular responses, such as cell cycle arrest and apoptosis (39). As stress-induced phosphorylation at Ser15 is required for p53-mediated activities (39), we determined the effect of radiation and rapamycin on the level of phosphorylated p53 in the cells (Fig. 5A). Phosphorylation of p53 at Ser15 was not detected in unirradiated cells but increased dramatically following radiation. Interestingly, although rapamycin alone did not lead to increased phosphorylation of p53, when added immediately following radiation, it enhanced the radiation effect on the phosphorylation of p53 as reflected by increased ratio of phosphorylated p53 to total p53 and by increased total level of phosphorylated p53. These results show that inhibition of rapamycin-sensitive functions in irradiated cells participates in the regulation of DNA damage response and that the consequence of rapamycin treatment may differ between irradiated and unirradiated cells.

We then determined the effect of rapamycin on the survival of the cells to radiation treatment during 48 hours following initiation of treatment (Fig. 5B). Although the outcome of rapamycin or radiation treatment was a decreased cell growth, combined treatment of radiation and rapamycin was the only treatment that decreased cell number below their numbers at the initiation of treatment. In colony survival assay (Fig. 5C), which better predict the long-term effect of anticancer treatment on tumor survival, rapamycin alone did not affect cell survival but increased their sensitivity to a low radiation dose (3 Gy). At 10 Gy, no surviving colonies were detected; obviously, rapamycin could not lead to enhancing effect. Taken together, these results show that rapamycin treatment enhances clonogenic death following a low radiation dose and may facilitate cell death following a high radiation dose.

Discussion

The dephosphorylation of p70S6K and 4E-BP1 in irradiated MCF-7 cells suggests that mTOR pathway is inhibited in these cells. The question of how mTOR pathway is inactivated by radiation awaits further studies. Kumar et al. (32) reported that overexpressed c-abl phosphorylates and inactivates overexpressed mTOR in irradiated MCF-7 cells. It remains to be seen if endogenous mTOR responds to radiation in the same manner. Yet, our data showing that radiation decreased the level of phosphorylation of mTOR's autophosphorylation site and increased its nuclear immunolocalization in a manner similar to that induced by rapamycin suggest that alteration in mTOR molecule, its intracellular localization, and/or its cellular localization participate in the observed inactivation of its downstream effectors. It is possible that in some cell types incongruous distribution of mTOR and its substrates will result in alteration in the activity of mTOR pathway. For example, under some circumstances, mTOR and p70S6K may be segregated in the nuclei, whereas 4E-BP1 remains in the cytoplasm (40), which in turn may lead to increased dephosphorylation of 4E-BP1 relative to p70S6K and to a diminished effect on S6 phosphorylation as seen in our irradiated cells.

The reciprocal relationship between mitochondria metabolism and the activity of rapamycin-sensitive pathway has been described before. Mitochondria dysfunction leads to inhibition of mTOR (41), and inactivation of p70S6K results in dephosphorylation of bad, potentially leading to increased interaction between bad and bcl-x or bcl-2, to mitochondrial damage and cell death (42). In addition, rapamycin was shown to diminish the protective effect of myristoylated Akt on \( \Delta \Psi_m \) of murine pro-B-cell line deprived of interleukin-3 (43).

Cell death is most commonly associated with the collapse of \( \Delta \Psi_m \) (44). However, in some instances, cell death is associated with increased \( \Delta \Psi_m \) that usually precedes caspase activation and total collapse of \( \Delta \Psi_m \) and serves as an early sign of increased apoptotic

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**Figure 3.** Radiation and rapamycin increased \( \Delta \Psi_m \). Treatment with rapamycin, radiation, or both, incubation with inhibitors of mitochondrial oxidative phosphorylation, and staining with JC-1 were done as described in Materials and Methods. The vehicle DMSO was added to control and irradiated cells at a final concentration of 0.01%. A, distribution of cells from control and treated populations according to their red-to-green fluorescence ratio: cells were stained with JC-1 48 hours following initiation of treatment with 10 Gy, 50 nmol/L rapamycin, or both. Numbers at the top right are mean red-to-green ratio of the respective cell population. Numbers above bars at the bottom right represent the fraction of cell population (in %) in each treatment with red-to-green ratio above 1.5. Representative experiment that was reproduced several times. At 48 hours following initiation of treatment, the fold increase in mean red-to-green ratio in irradiated or rapamycin-treated cells relative to untreated control was 1.48 ± 0.06 (n = 10) and 1.82 ± 0.12 (n = 7), respectively (mean ± SE). In cells receiving both rapamycin and radiation treatment, red-to-green fluorescence ratio increased 2.14 ± 0.05-fold (n = 3) relative to control. B, the effect of radiation and rapamycin on \( \Delta \Psi_m \) is dose dependent: cells treated with the vehicle alone or with increasing doses of rapamycin (nmol/L) or radiation (Gy) were stained with JC-1 as described in Materials and Methods. Columns, mean red-to-green ratio of triplicate samples; bars, SE. *, **, P < 0.01, significant differences between experimental groups and control. C, the effect of radiation and rapamycin on \( \Delta \Psi_m \) is time dependent: cells were treated either with the vehicle alone, 10 Gy, 50 nmol/L rapamycin, or both radiation and rapamycin. Their mean red-to-green fluorescence ratio was determined as described in Materials and Methods. Columns, mean red-to-green ratio of triplicate samples; bars, SE. *, **, P < 0.01.
tendencies (26). Increased $\Delta \Psi_m$ can result from reversed activity of the mitochondrial $F_oF_1$ATPase caused by reduced ADP transport into the mitochondria. The resulting increase in $\Delta \Psi_m$ then inhibits the electron transport through the cytochromes, thus leading to interruption of mitochondria respiration and to accumulation of NADH, swelling of the matrix, and, eventually, rupture of the outer mitochondria membrane (26, 45).

Similarly, the observed increase in the fraction of cells with highest $\Delta \Psi_m$ in our experiments was mediated, at least in part, by the reversed activity of $F_oF_1$ATPase as indicated by the depolarizing effect of oligomycin. Interestingly, although the combined treatment of rapamycin and radiation led to increased cell death, it did not seem to induce accumulation of cells with decreased $\Delta \Psi_m$ but instead further enhanced the increase in cell fraction with hyperpolarized mitochondria.

A direct role for Akt, one of mTOR upstream regulators, in mammalian autophagy has been shown before (46). However, our experiments with GFP-LC3 and mTOR constructs showed in a direct fashion the role of mTOR itself as a negative regulator of autophagy in mammalian cells and thus lead to the conclusion that radiation-induced inactivation of mTOR pathway mediates radiation-induced autophagy. What are the downstream effectors

**Figure 4.** Radiation and rapamycin treatment induce autophagy: (A) autophagic vacuoles in irradiated and rapamycin-treated cells. Cells were fixed and processed for electron microscopy viewing 48 hours following treatment with 10 Gy or 50 nmol/L rapamycin. a and b, autophagic vacuoles in irradiated cells; c and d, autophagic vacuole vesicles in rapamycin-treated cells. Bar, 160 nm (a), 400 nm (b), 250 nm (c), and 190 nm (d). B, radiation and rapamycin increased the level of LC3-II. Control and irradiated cells were harvested 24 hours following radiation. Cells treated with 10 and 50 nmol/L rapamycin or the vehicle were harvested 16 hours following treatment. LC3-I and LC3-II were detected in Western blots as described in Materials and Methods. Differences in LC3 electrophoretic patterns of C and D reflect different harvest time rather than a vehicle effect. C, LC3 associates with ring- and dot-like structures following irradiation and rapamycin treatment. Controls, irradiated, 50 nmol/L rapamycin, and vehicle-treated cells were fixed, stained with anti-LC3, and viewed with fluorescence microscope as described in Materials and Methods. Arrows in insets, ring-like structures. Bar, 10 $\mu$m. D, inhibition of mTOR pathway is sufficient to induce autophagy in MCF-7 cells. Cells were cotransfected with GFP-LC3 and either empty vector (V), WT, or dominant-negative N2343K (NK) HA-tagged mTOR as described in Materials and Methods. Twenty-four hours following the transient transfection, the cells were stained with anti-HA to detect the expression of transfected mTORs. The nuclei were stained with DAPI. Quantification of autophagy was conducted in ~25% of the cells expressing both HA-mTOR and GFP-LC3. These cells were identified by their red fluorescent HA-mTOR and green fluorescent GFP-LC3. As can be seen in the fluorescent images and Table 1, expression of the dominant-negative mTOR (N2343K) increased the appearance of green fluorescent autophagosomes. Bar, 10 $\mu$m.
of mTOR that regulate autophagy is still not clear (35). Also not clear is how other regulators of mammalian autophagy, such as PI3K class III and phosphorylated elf2α, are related to inactivation of mTOR pathway. Our analysis of the rapamycin and radiation effect on p70S6K and S6 phosphorylation suggests that dephosphorylation of these proteins may not be required for autophagy to occur. Indeed, genetic experiments in Drosophila have shown recently that in fact p70S6K activity promotes rather than suppresses autophagy (47). It has been shown that changes in mitochondria metabolism following stress signals can trigger autophagy (48, 49). Our experiments show that, whereas development of radiation-induced increase in ΔΨm lagged behind development of autophagy, 10 nmol/L rapamycin had a saturating effect on development of ΔΨm but induced low levels of autophagy. These results suggest that increased ΔΨm does not mediate autophagy. However, it is quite possible that other mitochondrial changes, not detected by our measurements, do contribute to the autophagic response in these cells.

The ability of rapamycin to modulate cellular response to stress signals has been shown in many cellular systems (16, 18, 19). Thus, increased sensitivity to stress by rapamycin was taken as evidence that activity of mTOR pathway protects against stress, whereas increased survival was taken as evidence that activity of mTOR pathway enhances damage (16, 18, 19). Our experiments show that

### Table 1. Quantification of autophagy in cells expressing empty vector, HA-tagged WT, or dominant-negative N2343K mTOR constructs

<table>
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NOTE: Autophagosomes were counted in cells expressing both GFP-LC3 and HA-tagged mTOR constructs and therefore showed both green and red fluorescence (see Fig. 4D). Quantification in cells transfected with empty vector was conducted in the green fluorescent cells. The majority (~80%) of the cells transfected with WT or empty vector possessed cells with <10 autophagous bodies, whereas transfection with N2343K doubled the fraction of cells that develop large number (~10) of autophagous bodies. The experiment was reproduced once with same results.

**Figure 5.** Inhibition of rapamycin-sensitive pathway in irradiated cells increases the level of p53 (P-Ser15) and cellular sensitivity to radiation. A, rapamycin enhances phosphorylation of p53 in irradiated cells: cells treated with 10 Gy or 50 nmol/L rapamycin, exposed to both radiation and rapamycin (50 + 10Gy), or treated with the vehicle alone were harvested 48 hours later and analyzed for their content of p53 and phosphorylated p53 (P-p53) as described in Materials and Methods. The numbers represent fold increase in ratio of P-p53 to total level of p53 in cells exposed to both radiation and rapamycin relative to cells exposed to radiation alone. In two separate experiments, the P-p53/p53 ratio was 1.26- and 1.5-fold higher in cells treated with both rapamycin and than in cells exposed to radiation alone. The total level of P-P53 in cells treated with both radiation and 50 nmol/L rapamycin was also 1.3- and 1.2-fold higher than that found in cells treated with radiation alone. P-p53 was not detected in cells treated with rapamycin alone or in controls. B, rapamycin enhances cell death following radiation. Experimental conditions are as in (A). Attached cell population was harvested and counted with hemocytometer 48 hours following initiation of treatment. Greater than 95% of the attached population from all treatments excluded trypan blue. An accumulation of floating dead cell fragments was observed in tissue culture dishes receiving the combined treatment of rapamycin and radiation. Columns, mean of triplicate samples from one experiment that was reproduced twice with similar results; bars, SE. **, *P < 0.01, significant differences between experimental groups and control; *, *P < 0.05, significant difference between cell numbers in group treated with both rapamycin and radiation and the number of cells at the time of treatment initiation (C). C, rapamycin treatment decreases clonogenic survival of irradiated cells: cells were plated for clonogenic assay as described in Materials and Methods. Ten days following initiation of treatment, the cells were stained with crystal violets and colonies containing >50 cells were counted as described in Materials and Methods. 3Gy, cells irradiated with 3 Gy; 50 + 3Gy, cells treated with both rapamycin and radiation. **, *P < 0.01, significant difference between surviving fraction of cells treated with radiation or radiation and rapamycin and the groups treated with rapamycin or the vehicle alone; *, *P < 0.05, significant difference in surviving fraction between the group treated with radiation and the group treated with both rapamycin and radiation.
References


4. KIM DH, SARASSOV DD, ALI SM, ET AL. G1/S, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol Cell 2003;11:895–904.


25. SALVINI S, ARIDIZZONI A, FRANCESCHI C, COSARIZZA A. J1C-1, but not DIO3(S) or rhodamine 123, is a reliable fluorescent probe to assess changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett 1997;411:77–82.


36. SADOH M, YODO meiner foresto. for the preparation of the figures. Cancer Res 2005;65: (23), December 1, 2005 11070 www.aacrjournals.org

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